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Purified Outer Membranes of *Serpulina hyodysenteriae* Contain Cholesterol

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We have isolated outer and inner membranes of *Serpulina hyodysenteriae* by using discontinuous sucrose density gradients. The outer and inner membrane fractions contained less than 1 and 2%, respectively, of the total NADH oxidase activity (soluble marker) in the cell lysate. Various membrane markers including lipooligosaccharide (LOS), the 16-kDa outer membrane lipoprotein (SmpA), and the C subunit of the F_1F_0 ATPase indicated that the lowest-density membrane fraction contained outer membranes while the high-density membrane fraction contained inner membranes and that both are essentially free of contamination by the periplasmic flagella, a major contaminant of membranes isolated by other techniques. The outer membrane fractions (ρ = 1.10 g/cm^3) contained 0.25 mg of protein/mg (dry weight), while the inner membrane samples (ρ = 1.16 g/cm^3) contained significantly more protein (0.55 mg of protein/mg [dry weight]). Lipid analysis revealed that the purified outer membranes contained cholesterol as a major component of the membrane lipids. Treatment of intact *S. hyodysenteriae* with different concentrations of digitonin, a steroid glycoside that interacts with cholesterol, indicated that the outer membrane could be selectively removed at concentrations as low as 0.125%.

Swine dysentery, caused by *Serpulina* (formerly *Treponema*) *hyodysenteriae* (9, 10), is a highly infectious, acute to chronic disease that can result in a debilitating mucohemorrhagic diarrhea and death. The first stage of the disease is spirochetal colonization and proliferation in the enteric epithelium, resulting in a significant decrease in the absorption of nutrients, ions, and water (12). However, the spirochetes do not invade beyond the lamina propria, and putative virulence factors, such as hemolysins and lipooligosaccharide (LOS), probably cause the cellular damage (16, 21–24, 35). Because a close association of the bacteria with the intestinal lining is critical in the development of disease, surface components of the spirochetes must play a pivotal role in the process.

Previously, different groups have attempted to isolate outer membranes (OMs) from *S. hyodysenteriae* with less than favorable results. This was mainly due to the contamination of OM fractions by periplasmic flagella. For example, Joens et al. (11) found seven major proteins, ranging from 42 to 32 kDa in size, in OMs isolated from *S. hyodysenteriae* B204 by a Sarkosyl extraction technique. However, six of the seven bands appeared to be derived from the periplasmic flagella. Similarly, Chatfield et al. (5) and Wannemuehler et al. (39) found that the majority of the putative OM proteins isolated with detergent ranged in size from 45 to 29 kDa and strongly resembled the protein profile observed for the flagellar proteins. These data suggested that membranes isolated by these techniques were heavily contaminated by flagella. Therefore, a more effective technique is needed to better define the OM of *S. hyodysenteriae.*

We have developed a membrane separation technique that permits the isolation of *S. hyodysenteriae* OMs and inner membranes (IMs) with minimal contamination by flagella. Analysis of the OMs indicated that they are quite interesting. First, the OM of *S. hyodysenteriae* has a lower density than the IM, which is very unusual for bacteria. In addition, the OM contains significant levels of cholesterol. Previously, *Mycoplasma* species (6), *Borrelia hermsii* (15), and *S. hyodysenteriae* (30, 33) had been described as having sterols in their membranes; however, the distributions of the cholesterol in various membrane fractions in *S. hyodysenteriae* and *B. hermsii* were not known. The data presented in this report suggest that the OM contains virtually all of the cell-associated cholesterol. By defining the components of the OM of *S. hyodysenteriae*, we will gain a better understanding of the role of these surface structures in the pathogenesis of swine dysentery.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Stock cultures of *S. hyodysenteriae* B204 were provided by T. B. Stanton, National Animal Disease Center, Ames, Iowa. *S. hyodysenteriae* cells were cultured in brain heart infusion (Difco Laboratories, Detroit, Mich.) supplemented with 5% heat-inactivated normal rabbit serum (Atlanta Biologicals, Norcross, Ga.) under an N_2-CO_2-O_2 (85:10:5, vol/vol/vol) atmosphere. Cells were enumerated by dark-field microscopy as described by Miller (18). Frozen stocks were prepared by resuspending cell pellets in a solution of sterile 30% glycerol in brain heart infusion medium that was then flushed with N_2 for 3 min. *Borrelia burgdorferi* B31 (passage 5) was grown as previously described (3).

Membrane separation technique for *S. hyodysenteriae.* The technique for the isolation of IMs and OMs was based on the method of Bledsoe et al. (3) but with extensive modifications (Fig. 1). All sucrose solutions were made in 20 mM HEPES buffer containing 50 mM NaCl (pH 7.6) (buffer I), and the concentrations are expressed as percentages (wt/vol). All procedures were performed at 4°C unless stated otherwise. When a 4-liter culture reached a cell density of 5×10^8 cells/ml, bacteria were harvested by centrifugation (10,000 × *g* for 10 min). The cell pellet was collected, washed twice with 100 ml of buffer I, and resuspended in 30 ml of buffer I containing 10% sucrose and 2 mM EDTA. In some experiments, 500 μg of Pefabloc SC (Boehringer Mannheim, Indianapolis, Ind.) per ml was added to inhibit protease activity. DNase type 1 and RNase type A (40 μg each) were added, and the cell suspension was passed through a 22-gauge needle to break up cellular aggregates. The cells were lysed by two passages through a cold French pressure cell (15,000 lb/in^2), and cell debris was removed

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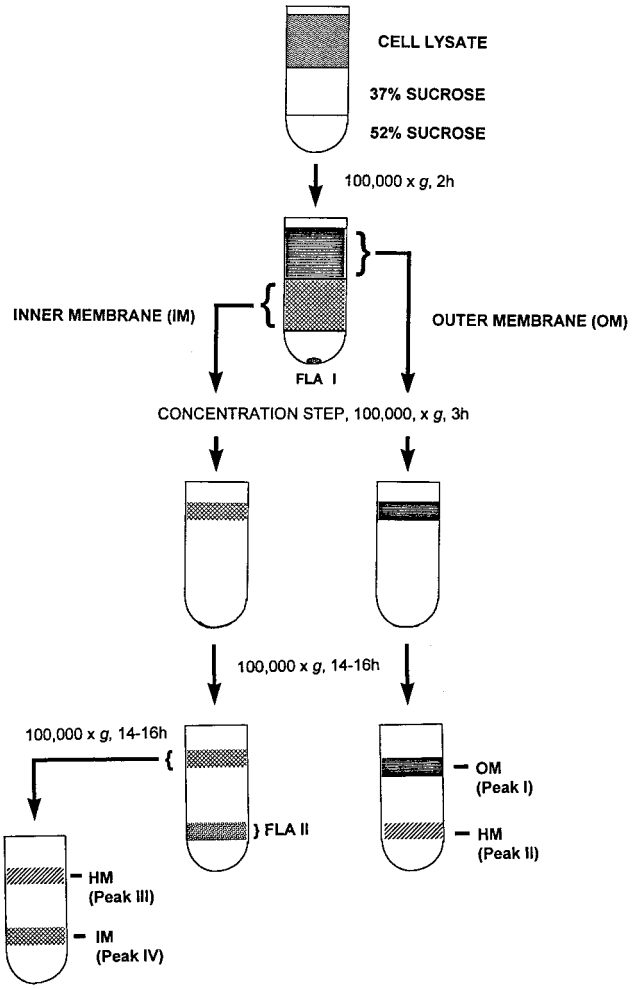

FIG. 1. Schematic drawing of the membrane separation technique.

by centrifugation (12,000 × *g* for 15 min). A 1-ml portion of the supernatant (cell lysate) was removed and stored at −20°C for future analysis.

The remaining cell lysate in 10% sucrose was divided in half, and each portion was gently layered onto a step gradient consisting of 8 ml of 52% and 14 ml of 37% sucrose (Fig. 1). The gradients were subjected to centrifugation with a Beckman SW28 rotor (100,000 × *g* for 2 h). The material that moved into the lower, 52% sucrose layer was diluted to 40 ml with buffer I, harvested by centrifugation (100,000 × *g* for 3 h), and resuspended in 500 μl of buffer I (Fig. 1, FLA I). This fraction contained mainly flagella.

The middle, 37% sucrose layer (high-density fraction, putative IM enrichment) was collected, transferred to a clean tube, and brought to a final volume of 35 ml with buffer I. A 2-ml volume of 30% sucrose was placed at the bottom of the tube, and each high-density enrichment was concentrated by centrifugation (100,000 × *g* for 3 h). The material that moved into the 30% sucrose pad was collected, diluted with 2 ml of buffer I to a final sucrose concentration of 17.5%, layered directly on a discontinuous gradient (consisting of 3 ml of 60%, 4 ml of 55%, 16 ml of 50%, 4 ml of 40%, and 3 ml of 30% sucrose), and centrifuged (100,000 × *g* for 14 to 16 h). Two distinct bands, an upper membrane band (putative IMs) and a lower flagellum band (Fig. 1, FLA II), were observed. The flagellum band was collected, diluted in 35 ml of buffer I, and harvested by centrifugation (100,000 × *g* for 3 h). The membrane enrichment band was carefully harvested, layered on a second discontinuous gradient consisting of 5 ml of 55%, 3 ml of 50%, 16 ml of 40%, 4 ml of 30%, and 3 ml of 25% sucrose, and centrifuged (100,000 × *g* for 14 to 16 h). Two distinct bands were observed, an upper, intermediate-density band (putative hybrid membrane [HM], an intermediate-density fraction that contains both IM and OM components), and a lower, high-density band (putative IMs). Fractions (1 ml) were collected from the bottom of each tube with gradient fractionator (Hoefer Scientific, San Francisco, Calif.).

The upper layer (in 10% sucrose) from the original step gradient (low-density fraction, putative OM enrichment) was collected, transferred to a clean centrifuge tube, and brought to a final volume of 35 ml with buffer I. A 2-ml volume of 25% sucrose was gently placed at the bottom of the tube, and the membrane enrichments were concentrated by centrifugation (100,000 × *g* for 3 h). The material that moved into the pad was collected, diluted with 2 ml of buffer I to a final sucrose concentration of 12.5%, layered directly on a discontinuous gradient (consisting of 4 ml of 60%, 3 ml of 50%, 19 ml of 36%, and 8 ml of 30% sucrose), and centrifuged (100,000 × *g* for 14 to 16 h). Two distinct bands were observed, an upper, low-density band (putative OMs) and a lower, intermediate-density band (putative HMs). Fractions (1 ml) were collected from these gradients as previously described.

The protein concentration (measured by monitoring the absorbance at 280 nm [A_{280}] and density (grams per cubic centimeter) of each fraction were measured with a Beckman DU 640 spectrophotometer and a Bausch & Lomb refractometer, respectively. The protein peaks were pooled, diluted with buffer I, and harvested by centrifugation (200,000 × *g* for 3 h). The membrane pellets were resuspended in buffer I and harvested a second time. The final membrane pellets were resuspended in 0.5 ml of 20% glycerol in buffer I and stored at −20°C. The protein concentration of the membrane samples was determined as described by Markwell et al. (17). Dry weights were determined by the method of Kotarski and Salyers (13).

Gel electrophoresis, immunoblotting, and sera. Membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Kotarski and Salyers (13). The molecular weight standards were purchased from Bio-Rad Laboratories, Richmond, Calif. (low-molecular standards). The proteins were visualized by staining with silver (19) or with Coomassie blue.

Immunoblotting was performed by the method of Towbin et al. (37). Proteins were transferred to nitrocellulose (0.45-μm-pore-size Protran membrane; Schleicher & Schuell, Keene, N.H.) with a Bio-Rad Trans Blot cell and visualized with Ponceau red (0.1% Ponceau red in 1% acetic acid), and the standards were marked. After probing, immunoreactive bands were detected with the enhanced chemiluminescence Western blotting detection kit from Amersham Life Science, Arlington Heights, Ill. Reactive proteins were quantitated by scanning the film with a scanning densitometer (no. 300A; Molecular Dynamics Corp., Sunnyvale, Calif.).

The anti-FlaB and FlaA antisera were obtained from M. Jacques, Faculté de médecine vétérinaire, Université de Montréal, Québec, Canada. The monoclonal antibody (MAb) against the *S. hyodysenteriae* serotype 2 LOS was obtained from L. A. Joens, Department of Veterinary Science, University of Arizona, Tucson, Az. (40). The MAb against the 16-kDa surface membrane lipoprotein (SmpA) was obtained from Richard Sellwood, Institute for Animal Health, Compton Newbury, England (29, 36). Rabbit polyclonal antiserum against the C subunit of the F_1F_0 ATPase complex of *Escherichia coli* was obtained from R. Fillingame, Department of Biological Chemistry, University of Wisconsin, Madison, Wis.

Two-dimensional nonequilibrium pH gradient electrophoresis, 2D-NEPHGE was performed by the method of Bledsoe et al. (3) with the following modifications. Briefly, a 50-μl sample containing 50 μg of membrane protein was precipitated by adding an equal volume of cold acetone. The sample was incubated for 30 min at 4°C and harvested by centrifugation (16,000 × *g* for 15 min at 4°C). The protein pellet was resuspended in 100 μl of 50% cold acetone, harvested by centrifugation, and dried at 37°C. The pellet was resuspended in 125 μl of sample solution (9 M urea, 2% 2-mercaptoethanol, 4% Nonidet P-40, 0.4% ampholytes [60% pH 3 to 10 ampholytes and 40% pH 2 to 11 ampholytes]) and incubated for 2 h at 26°C. The solubilized membrane samples were then applied to tube gels (0.2 by 16.0 cm), and electrophoresed for 2,800 V-h. The gels were equilibrated in SDS equilibration buffer (2% SDS, 125 mM Tris-HCl, 10% glycerol, 100 μl of 0.4% bromphenol blue [pH 6.8]), and the proteins were separated in the second dimension by SDS-PAGE (12.5% polyacrylamide). After electrophoresis, the proteins were visualized by silver staining as previously described.

Lipid analysis. IMs, OMs, and HMs (protein content, 100 μg) were collected by centrifugation (100,000 × *g* for 1 h at 4°C) and extracted with chloroform-methanol (2:1) at 26°C. The extracted membrane fractions were centrifuged (13,000 × *g* for 5 min at 26°C), the supernatant was saved, and the pellet was further extracted with chloroform-methanol (2:1). The organic extracts were combined and analyzed by thin-layer chromatography.

Lipids were separated by high-performance thin-layer chromatography on aluminum plates precoated with silica gel 60 (VWR Scientific). The solvent system used for plate development was chloroform-methanol-H_2O (65:35:5) or chloroform-acetone (85:15). Phosphate and lipid detection was done with molybdenum blue spray reagent as specified by the manufacturer (Sigma Chemical, St. Louis, Mo.). Phosphatidylglycerol, phospholipid mixture (phosphatidylethanolamine, phosphatidylinositol, and phosphatidylcholine), cholesterol, and cholestanol standards were obtained from Sigma. The total cholesterol for membrane fractions was determined enzymatically with the Abbott Spectrum high-performance diagnostic system.

Digitonin treatment of *S. hyodysenteriae*. Cultures (10 ml) of *S. hyodysenteriae* B204 and *B. burgdorferi* B31 were grown to a density of 5×10^8 cells/ml. Aliquots (1 ml) of the cultures were transferred to Eppendorf tubes, and the cells were harvested by centrifugation (7,000 × *g* for 10 min at 26°C). The cell pellets were resuspended in 1 ml of 50 mM NaCl–10% sucrose–20 mM HEPES (pH 7.6) (buffer II), harvested by centrifugation, and resuspended in 450 μl of buffer II.

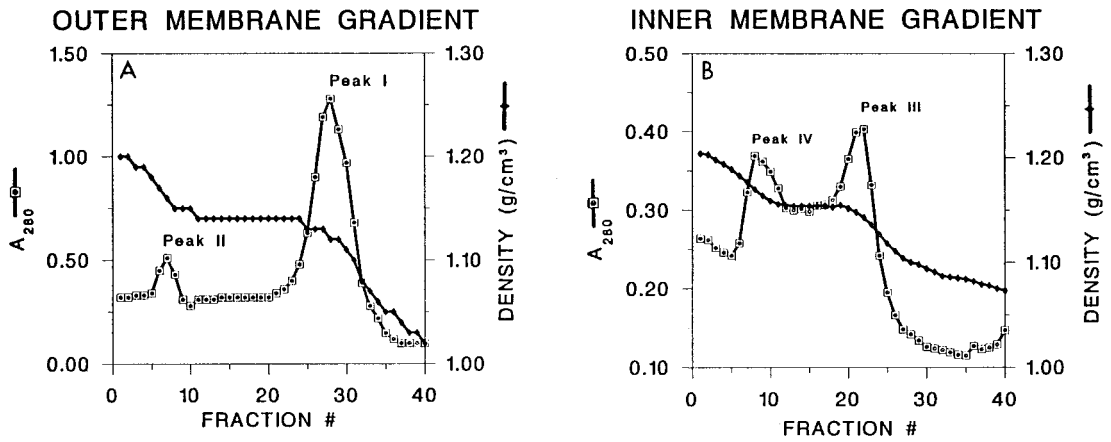

FIG. 2. Analysis of sucrose step gradients. Fractions (1 ml) were collected from inner and outer membrane sucrose gradients and analyzed for protein (A_{280}) and density (grams per cubic centimeter). (A) Peak I (fractions 23 to 27) and peak II (fractions 6 to 8) from the OM gradient were pooled and harvested by centrifugation. (B) Peak III (fractions 19 to 23) and peak IV (fractions 7 to 11) from the IM gradient were pooled and harvested by centrifugation.

Various amounts of a 5% suspension of digitonin (Sigma) solution in buffer II were added to each tube, and the reaction mixture volume was increased to 500 μl with buffer II. The resulting digitonin concentrations ranged from 0 to 0.5%. The tubes were incubated for 1 h at 26°C with gentle agitation, and the cells were harvested by centrifugation (7,000 × *g* for 10 min at 26°C). The treated cells were resuspended in 250 μl of buffer II, 10-μl aliquots were negatively stained (7), and samples were examined under a JEOL C× 100 electron microscope at 80 keV.

Enzyme assays. The NADH oxidase activities in subcellular fractions were determined by measuring the rate of NADH oxidation (decrease in A_{340}) as described by Stanton and Jensen (34). Briefly, all measurements were done in duplicate at 24°C with a Beckman DU 640 spectrophotometer equipped with an automatic cuvette sampler. The molar extinction coefficient for NADH was 6.22×10^3 M^{-1} cm^{-1} at 340 nm.

RESULTS

Membrane separation technique. Initially, we attempted to separate OMs and IMs from *S. hyodysenteriae* B204 by the method described by Bledsoe et al. (3) for the purification of membranes from *B. burgdorferi*. When this method was used for strain B204, the membrane fractions were heavily contaminated with endoflagella as determined by SDS-PAGE (data not shown). The highest-density material from the sucrose gradients (ρ = 1.23 to 1.25 g/cm^3) consisted primarily of endoflagella, while other fractions (ρ = 1.10 to 1.16) contained fewer flagellar components and significantly more membrane vesicles, as determined by electron microscopy (data not shown). We took advantage of the differences in density between the membranes and endoflagella to develop a technique that effectively separated OM and IM fractions essentially free of endoflagella. The final procedure (Fig. 1) is described in detail in Materials and Methods.

The densities of the protein peaks varied from 1.10 to 1.16 g/cm^3. Interestingly, the lowest-density peak from the gradients (later identified as OMs) (Fig. 2A, peak I), had densities that were much lower than expected, ranging from 1.09 to 1.12 g/cm^3. The highest-density peak (later identified as IMs) (Fig. 2B, peak IV) had densities ranging from 1.15 to 1.17 gm/cm^3. HMs were isolated from both gradients (Fig. 2, peaks II and III) and had intermediate densities from 1.13 to 1.15 gm/cm^3. The flagellar fractions (Fig. 1, FLA I and FLA II) recovered during the initial stages of the separation had the highest density (1.22 g/cm^3). In addition, >95% of the total flagella from the cell lysate were recovered in these fractions.

Fractions that included each of the peaks (Fig. 1, peaks I to IV) were pooled and concentrated by centrifugation. Protein analysis of these samples indicated that the low-density peak (Fig. 2A, peak I) had 0.25 mg of protein per mg (dry weight) of membrane isolated. The high-density peak (Fig. 2B, peak IV), on the other hand, had 0.55 mg of protein per mg (dry weight). The smaller amount of protein in the low-density peak suggested that this fraction potentially represented the OM fraction from *S. hyodysenteriae*. This observation was consistent with previous freeze-fracture analysis that indicated that *S. hyodysenteriae* had fewer transmembrane particles in its OM than *E. coli* did (38). The measured amount of protein in the OM of *S. hyodysenteriae* is larger than the 0.15 mg of protein per mg (dry weight) reported for *B. burgdorferi* (3). This is also consistent with the freeze-fracture data that suggested that *S. hyodysenteriae* OMs had more transmembrane particles than did *B. burgdorferi* or *Treponema pallidum* (38).

Membrane markers. Several investigators have identified and characterized major antigens and other components of the OMs of *S. hyodysenteriae* that may be involved in virulence or host immunity. Monoclonal antibodies (MAbs) raised against two OM components were used to probe the membrane fractions isolated from the sucrose gradients. First, when membrane fractions were probed with a MAb directed against SmpA (29), the lowest-density peak (ρ = 1.10 g/cm^3) reacted the most strongly at 16 kDa (Fig. 3A, lane OM), suggesting that this peak contained significant amounts of OMs. An equally strong reaction at 16 kDa was observed with the intermediate-density peak (lane HM). No measurable reaction was observed with the high-density peak (lane IM), suggesting that this peak contained mostly IMs.

Some of the markers that have been used to identify OMs from other spirochetes have led to confusing results and erroneous information (3, 4, 25). Therefore, a second OM marker was used to tentatively identify various membrane fractions. When membrane fractions were probed with MAb to the LOS (an OM marker) from *S. hyodysenteriae* B204 serotype 2 (11, 21, 31), the band obtained ranged from 16 to 26 kDa (Fig. 3). This diffuse band was similar to those observed previously for LOS from *S. hyodysenteriae* B204 (35). The strongest reaction was observed with the lowest-density peak (Fig. 3B, lane OM) supporting the original observation that this peak was primarily OM. The intermediate-density peak reacted almost as strongly (lane HM), while the high-density peak reacted very weakly (lane IM).

To confirm the localization data, membrane fractions were probed with polyclonal antiserum to the C subunit of F_1F_0

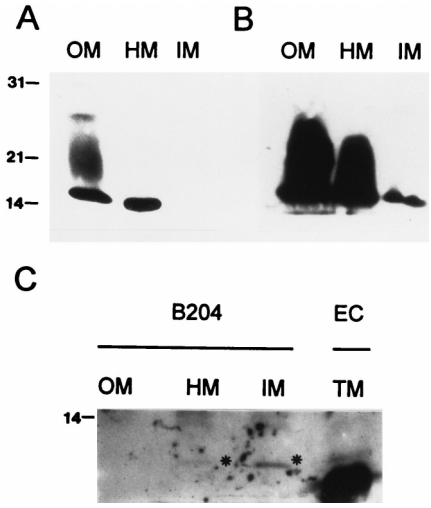

FIG. 3. Localization of membrane markers. Immunoblots of membrane fractions probed with MAb to SmpA (A), MAb to serotype 2 LOS (B) or polyclonal antiserum raised against the 9.5-kDa C subunit of F_1F_0 ATPase from *E. coli* (C). The lanes contained OM, HM, or IM from *S. hyodysenteriae* B204, or total membranes (TM) from *E. coli* (C). Each lane contained 30 μg of protein. Reactive proteins were visualized by enhanced chemiluminescence (Amersham Corp.). The asterisks in the gel in panel C indicate reactive bands in the hybrid and inner membrane fractions. The numbers on the left indicate the relative mobilities of molecular mass standards.

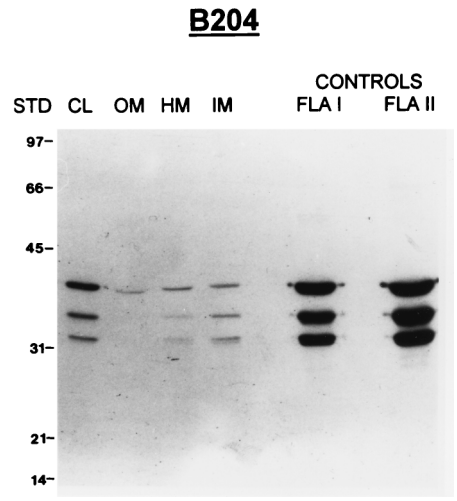

FIG. 4. Localization of flagellar proteins. An immunoblot of subcellular fractions probed with antisera raised against FlaB as shown. Lanes: STD, molecular mass standards; CL, cell lysate; OM, outer membranes (Fig. 2A, peak I); HM, hybrid membranes (Fig. 2B, peak III); IM, inner membranes (Fig. 2B, peak IV); FLA I and FLA II, periplasmic flagella (Fig. 1). Reactive proteins were visualized by enhanced chemiluminescence.

ATPase (an IM marker) of *E. coli*. A 10-kDa protein in the high-density peak reacted the most strongly with antiserum (Fig. 3C, lane IM), confirming this fraction as IM, while no band was detected in the low-density fraction, identifying this fraction as OM. A 9.5-kDa band was detected in the control lane containing total membranes from *E. coli* (lane TM). These data also strongly suggest that the low-density peak is OM while the high-density peak is IM.

Further analysis of membrane fractions. First, to measure the contamination of the membrane fractions by soluble protein, membrane fractions were assayed for NADH oxidase activity (a soluble marker) (34) (Table 1). The OMs contained 2% of the total NADH oxidase activity from the cell lysate, while the IMs contained 1%. These data indicated that the various membrane samples showed only minor contamination by soluble protein.

To measure the levels of cross-contamination among various membrane samples, OMs, HMs, and IMs of *S. hyodysenteriae* B204 were probed with MAb to SmpA, MAb to serotype 2 LOS, and polyclonal antiserum to the F_1F_0 ATPase C subunit (Fig. 3). Reactive bands were quantitated by densitometry, and the results are shown in Table 1. Analysis with LOS as an OM marker indicated 3% cross-contamination of IMs by OMs. The anti-LOS MAb reacted with a diffuse band ranging from 14 to 26 kDa. Joens et al. (11) had noted a similar reactive pattern for LOS from *S. hyodysenteriae* serotype 2 when membranes were probed with anti-LOS polyclonal serum. Similar results were obtained with the MAb to SmpA, which demonstrated <1% cross-contamination. More than 97% of these markers were measured in the OM and HM fractions. Less than 1% of the IM marker (F_1F_0 ATPase) was detected in the OM fraction, and 96% localized to the IM and HM fractions. These data indicate that there were very low levels of cross-contamination between the OM and IM fractions.

Previous attempts by other groups to purify OMs from *S. hyodysenteriae* have been hindered by the periplasmic flagella. These complex structures are a major contaminant of membranes isolated by other techniques (11, 39, 40). To evaluate how effectively the membrane separation technique eliminated periplasmic flagella from isolated samples, membrane fractions (as well as the flagellar fractions FLA I and FLA II [Fig. 1]) were probed with antiserum to FlaB (Fig. 4). The antiserum reacted with three bands of 39, 35, and 33 kDa, which represent the three different forms of FlaB. The OMs, HMs, and IMs showed little contamination by FlaB (Fig. 4, lanes OM,

TABLE 1. Physical and biochemical characteristics of membrane fractions

Fraction (density) [g/cm^3])	% of:					Amt of cholesterol (μg/ml)
	Soluble marker (NADH oxidase)[a]	OM markers		IM marker (F_1F_0 ATPase)[b]	Fla marker (FlaB)[b]	
		16-kDa SMLP[b,c]	LOS[b]			
OM (1.10)	2	52	60	<1	2	380
HM (1.14)	2	47	37	26	3	130
IM (1.16)	1	<1	3	70	5	30
Fla (1.22)	<1	<1	2	<1	90	ND[d]

[a] Determined as a percentage of the total from cell lysate.
[b] Determined by densitometry and expressed as a percentage of the total.
[c] SMLP, 16-kDa surface membrane lipoprotein.
[d] ND, not determined.

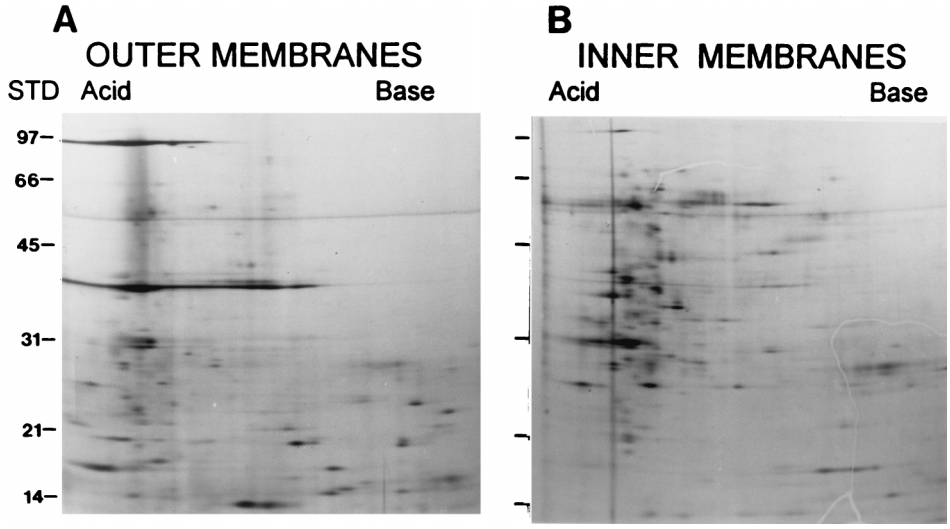

FIG. 5. NEPHGE comparison of membrane fractions from *S. hyodysenteriae* B204. Proteins from OMs (A) and IMs (B) were compared by 2D-NEPHGE, with 50 μg of protein being separated on each gel. The proteins were visualized by silver staining. The numbers on the left indicate the relative mobilities of molecular mass standards.

HM, and IM). When the levels of contamination were measured by densitometry, 90% of the FlaB was isolated in the FLA I and FLA II fractions. Less than 2% of the total FlaB was detected in the OM fraction, while less than 5% localized to the IM fraction (Table 1). Very similar results were obtained when membrane and FLA fractions were probed with antiserum to FlaA (data not shown). These data indicate that the membrane purification technique permitted the isolation of OMs and IMs with minimal contamination by periplasmic flagella.

SDS-PAGE analysis of OMs and IMs of *S. hyodysenteriae* B204. The OM, HM, and IM fractions were analyzed by SDS-PAGE (12.5% polyacrylamide) (data not shown). The purified OM contained 30 to 35 polypeptides, most of which were unique to these membranes. The most prominent band was 39 kDa, and this protein did not appear to be related to any of the flagellar proteins, as determined by immunoblotting. When OM proteins were analyzed by SDS-PAGE with or without heat treatment, the 39-kDa protein showed altered mobility, suggesting that it was heat modified (data not shown). The IM contained 50 to 55 polypeptides with no predominant bands. However, most of the polypeptides appeared to be specific for the IM. The HMs contained polypeptides from both OMs and IMs. This is consistent with the hypothesis that HMs are fusions between OMs and IMs.

Analysis of OMs and IMs of *S. hyodysenteriae* B204 by 2D-NEPHGE. Purified *S. hyodysenteriae* B204 OMs and IMs were compared by 2D-NEPHGE, and the results are shown in Fig. 5. The separation of proteins from OMs (Fig. 5A) resulted in 40 to 45 discrete spots of different intensities. There were several more polypeptides than were observed by SDS-PAGE, and additional proteins were identified which migrated with proteins with similar molecular masses when OMs were analyzed by SDS-PAGE. The IM (Fig. 5B) protein separation resulted in 55 to 60 discrete spots of different intensities. Again, additional spots were observed compared to the number observed by SDS-PAGE. Seven to ten polypeptides were common to both IMs and OMs, in particular, polypeptides with apparent molecular masses of 31, 29, 28, 25, 24, and 22 kDa. Polypeptides common to both OMs and IMs were described for purified *B. burgdorferi* membranes (e.g., OspA [3]). Therefore, IM and OM markers better reflect the purity of isolated fractions. It should also be noted that no protein with an apparent molecular mass consistent with serum albumin (66 kDa) was observed in the OMs or IMs when analyzed by 2D-NEPHGE (Fig. 5). This indicated that the major contaminant from the culture media (albumin) had been successfully removed by the initial washing of the intact bacteria, the passage of the membranes through the sucrose gradients, and the successive washing of the membranes after isolation from the gradients and before analysis by 2D-NEPHGE.

Analysis of the membrane lipids. Stanton and Cornell (30, 33) had previously reported that *S. hyodysenteriae* required cholesterol for growth and that the sterol was incorporated into membranes and was not metabolized. Also, it appeared that all of the cholesterol taken up by the bacteria localized to the membrane fraction. To further investigate the localization of cholesterol in *S. hyodysenteriae*, we extracted the lipids from OM, HM, and IM fractions and analyzed the samples by thin-layer chromatography. The results are shown in Fig. 6. The

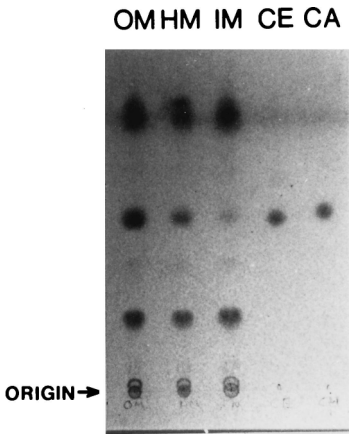

FIG. 6. Analysis of the lipids from membrane fractions from strain B204. Extracted lipids from OM, HM, or IM were separated by thin-layer chromatography and visualized as described in the text. The standards used were cholesterol (lane CE) and cholestanol (lane CA).

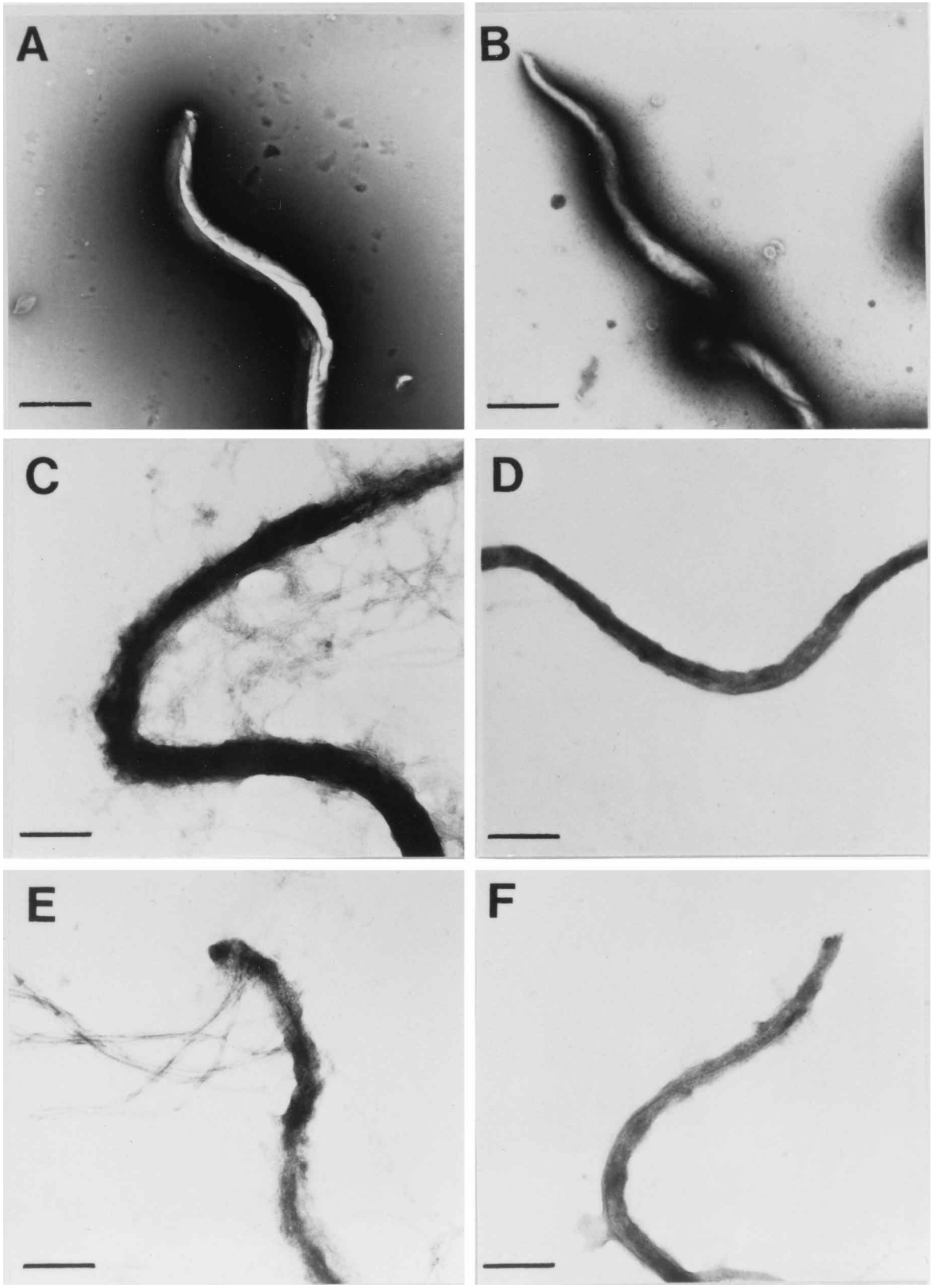

FIG. 7. Electron micrographs of digitonin-treated *S. hyodysenteriae* and *B. burgdorferi.* The cells were treated with increasing levels of digitonin, stained with 4% uranyl acetate, and examined by electron microscopy. (A, C, and E) *S. hyodysenteriae* B204 treated with 0, 0.125, and 0.250% digitonin, respectively. (B, D, and F) *B. burgdorferi* B31 treated with 0, 0.125, and 0.250% digitonin, respectively. Bars, 25 μM.

OMs and HMs contained a lipid that migrated with cholesterol (Fig. 6, lanes OM, HM, and CE) and not with cholestanol (also detected in *S. hyodysenteriae* cell extracts exposed to hydrogen [32]). When the levels of cholesterol were assayed biochemically in purified membrane fractions, 96% was isolated from the OM and HM fractions and 4% was isolated from the IM fraction (Table 1). The amount of cholesterol (380 μg/mg of protein) indicated that it was a major component of the OM, and the levels measured in the IM were within the limits for measured levels of cross-contamination of IMs by OMs. Other lipids identified were phosphatidylethanolamine and phosphatidylserine (data not shown).

Effect of digitonin on *S. hyodysenteriae*. If the OM of *S. hyodysenteriae* contained significant levels of cholesterol, as suggested by the extraction data, this membrane should be sensitive to agents that interact directly with sterols, such as the saponin digitonin. These compounds form a complex with cholesterol (20) and disrupt erythrocyte membranes (27, 28). To see what effect digitonin had on *S. hyodysenteriae*, cells were incubated with various concentrations of digitonin. The results are shown in Fig. 7. *B. burgdorferi* B31, which has an extremely labile OM (3), was used as a control to ensure that the observed effects were not due to the staining technique. When *S. hyodysenteriae* cells were incubated with 0.125% digitonin, the OM was severely compromised (Fig. 7C) and cells were observed to release OM fragments and the periplasmic flagella. When the digitonin concentration was increased, the OM was completely removed and cells began to undergo lysis (Fig. 7E). At digitonin concentrations greater than 0.5%, lysis was essentially complete. Digitonin at 0.125% had no effect on *B. burgdorferi* cells (Fig. 7D), and digitonin at 0.25% had a slight effect (Fig. 7F). This slight perturbation was not surprising considering the labile nature of the OM of *B. burgdorferi* (3, 4, 25, 38). Clearly, digitonin had a very dramatic effect on the outer surface of *S. hyodysenteriae.*

DISCUSSION

The technique that we have developed to isolate OMs and IMs from *S. hyodysenteriae* permits the isolation of OMs with minimal contamination from the periplasmic flagella (Table 1; Fig. 4) and with little cross-contamination among various membrane fractions. Previous attempts by other groups to isolate *S. hyodysenteriae* OMs by Sarkosyl extraction were plagued by contaminating periplasmic flagella (11, 39). In fact, the protein profile of Sarkosyl-extracted OMs most closely resembled the FLA I and FLA II fractions from the discontinuous sucrose gradients used to purify IM and OM. Thus, this technique gives a realistic representation of both the OM and IM of *S. hyodysenteriae*. In addition, we have been able to identify unusual characteristics of the OM of *S. hyodysenteriae*. First, the density of the OM was significantly lower than that of any previously described OMs from any gram-negative bacteria (3, 13). Second, this represents only the second bacterial membrane (*Mycoplasma* being the first) and the first bacterial OM that has been shown to contain cholesterol. Clearly, the OM of *S. hyodysenteriae* is unique.

Typically, a bacterial OM is more dense than the IM. This is believed to be due to several factors, including the types of lipids, the lipopolysaccharide, the capsule, and the protein composition of the OM. Spirochetal OMs have a reduced density compared to other bacteria with an IM and OM (3, 25). This is believed to be due to the decreased amounts of protein in the OM (3, 4, 38) and, for *T. pallidum* and *B. burgdorferi*, the lack of lipopolysaccharide (1, 2). Walker et al. (38) have shown, using freeze-fracture analysis, that *S. hyodysenteriae* has levels of transmembrane particles that are significantly higher than those of *T. pallidum*, *B. burgdorferi*, *B. hermsii*, or *T. denticola* (25, 26). In addition, Joens et al. (11) have identified a LOS from *S. hyodysenteriae*. Taken together, these data might suggest that the OM of *S. hyodysenteriae* would have greater density than that of *B. burgdorferi* or *T. pallidum*. However, we have found that the OMs of *S. hyodysenteriae* have the lowest density of any spirochetal OM ($\rho = 1.10$ g/cm^3) measured to date. Since the *S. hyodysenteriae* OM we have isolated contained significant amounts of cholesterol, we believe that this is contributing to the overall density of this membrane fraction.

Cholesterol is important for the healthy growth of *Serpulina*. In 1980, Lemcke and Burrows (14) gave the first report of the sterol requirement for the growth of *S. hyodysenteriae*. Interestingly, the addition of at least 1.25 μg of cholesterol per ml to the basal medium increased the viable counts approximately 1,000-fold. Stanton and Cornell (30, 33) have studied the metabolic fate of cholesterol and its nutritional role in *S. hyodysenteriae*. Experimentally, *S. hyodysenteriae* can assimilate approximately 4.4 μmol of cholesterol per 100 mg of cell dry weight, with >95% being incorporated into the membrane fraction. Interestingly, at the membrane level, most of the sterols were in the form of cholestanol (the ratio of cholestanol to cholesterol in cellular lipids was 19:1). Furthermore, in a separate experiment, cholestanol was found in the culture supernatant but none was detected in the uninoculated control media. However, these authors later demonstrated that the presence of H_2 in the growth media promotes the nonenzymatic reduction of cholesterol to cholestanol (34a). This could occur because *S. hyodysenteriae* produces significant amounts of H_2 as an end product of the metabolism of glucose.

We have been able to demonstrate that the OMs of *S. hyodysenteriae* contain cholesterol but no cholestanol. This could be because the *S. hyodysenteriae* cells were grown in the presence of 5% oxygen. Stanton (31) demonstrated that cell extracts of *S. hyodysenteriae* produce less H_2 and butyrate in the presence of 10% oxygen than under 100% nitrogen. Thus, under the growth conditions used for cells in the membrane separation experiments, less H_2 was available for the nonenzymatic reduction of cholesterol to cholestanol.

The role that cholesterol plays in the physiology and/or pathogenesis of *S. hyodysenteriae* can only be speculated. The differences in the physical properties of biological membranes containing cholesterol and those containing phospholipids alone is well documented. These include increased rigidity of the overall membrane structure, decreased solute permeability, and a broadening of the phase transition temperature between the gel (crystalline) and liquid (fluid) states (i.e., membrane fluidity is maintained over a wider temperature range) (reviewed in references 8 and 41). In addition, cholesterol may play a role in the uptake of fatty acids (e.g., cholesterol increases the uptake of oleic acid into the membrane of *Mycoplasma capricolum*) and in the conformation of membrane proteins (6). It is not known if some or all of these properties are important for the survival of *S. hyodysenteriae* in the intestinal tracts of host animals or for the pathogenesis of swine dysentery. However, because the OM directly interacts with infected tissue, defining the components of this structure will lead to a better understanding of the complex relationship between the bacterial pathogen and the host.

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